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Familial defective apolipoprotein B-100: Low density lipoproteins with abnormal receptor binding

(hypercholesterolemia/genetic disease/atherosclerosis/lipoprotein metabolism)

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ABSTRACT **Previous *in vivo* turnover studies suggested that retarded clearance of low density lipoproteins (LDL) from the plasma of some hypercholesterolemic patients is due to LDL with defective receptor binding. The present study examined this postulate directly by receptor binding experiments. The LDL from a hypercholesterolemic patient (G.R.) displayed a reduced ability to bind to the LDL receptors on normal human fibroblasts. The G.R. LDL possessed 32% of normal receptor binding activity (≈9.3 μg of G.R. LDL per ml were required to displace 50% of ^{125}I-labeled normal LDL, vs. ≈3.0 μg of normal LDL per ml). Likewise, the G.R. LDL were much less effective than normal LDL in competing with ^{125}I-labeled normal LDL for cellular uptake and degradation and in stimulating intracellular cholesteryl ester synthesis. The defect in LDL binding appears to be due to a genetic abnormality of apolipoprotein B-100: two brothers of the proband possess LDL defective in receptor binding, whereas a third brother and the proband's son have normally binding LDL. Further, the defect in receptor binding does not appear to be associated with an abnormal lipid composition or structure of the LDL: the chemical and physical properties of the particles were normal, and partial delipidation of the LDL did not alter receptor binding activity. Normal and abnormal LDL subpopulations were partially separated from plasma of two subjects by density-gradient ultracentrifugation, a finding consistent with the presence of a normal and a mutant allele. The affected family members appear to be heterozygous for this disorder, which has been designated familial defective apolipoprotein B-100. These studies indicate that the defective receptor binding results in inefficient clearance of LDL and the hypercholesterolemia observed in these patients.**

Low density lipoproteins (LDL) transport two-thirds of the plasma cholesterol in humans, and individuals with high plasma levels of these lipoproteins are predisposed to accelerated coronary disease (1). The plasma levels of LDL are determined in large part by LDL receptors, which bind LDL particles and remove them from plasma. The LDL receptors [or apoB,E(LDL) receptors] recognize and bind the protein moiety of LDL, apolipoprotein B-100 (apoB-100) (2).

The importance of apoB,E(LDL) receptors in the regulation of plasma LDL concentration is illustrated by the genetic disorder familial hypercholesterolemia (FH). Affected individuals lack or have defective apoB,E(LDL) receptors and develop hypercholesterolemia because of the impaired plasma clearance of the LDL (3). However, most individuals with elevated cholesterol levels apparently possess normal apoB,E(LDL) receptors [or apoB,E(LDL) receptors without detectable functional abnormalities] (4–6). Thus, in most cases of primary hypercholesterolemia, other abnormalities are responsible for elevated plasma LDL levels (6, 7). Recently, to examine the causes of other forms of primary hypercholesterolemia, the fractional catabolic rate of LDL was determined in a series of patients with moderate hypercholesterolemia (8). In some patients, simultaneous measurement of turnover rates of autologous and normal homologous LDL demonstrated fractional clearance rates for autologous LDL that were significantly lower than those for normal homologous LDL. These studies suggested that abnormal LDL might bind defectively to apoB,E(LDL) receptors; if so, this defective binding might account for the slow clearance of autologous LDL and for the development of moderate hypercholesterolemia (8).

In the present investigation, the ability of LDL from these same patients to bind to apoB,E(LDL) receptors was examined using an *in vitro* cell-surface binding assay. In one patient, the LDL had about one-third the receptor binding activity shown by normal LDL. It is probable that a structural defect in apoB-100 interfered with normal receptor binding, resulting in moderate hypercholesterolemia in this patient. The same abnormality was found in several of his first-degree relatives, indicating that this elevated LDL level is an inherited condition.

MATERIALS AND METHODS

Patient Material. Plasma was obtained from five patients known to have a slow clearance of autologous LDL compared with normal homologous LDL (8). For one patient (G.R.), plasma samples were obtained from four first-degree relatives (three brothers and one son).

Lipoproteins. Low density lipoproteins (*d* = 1.02–1.05 g/ml) were isolated from the plasma of these individuals by sequential ultracentrifugation at 59,000 rpm in a Beckman 60 Ti rotor (4°C) and washed by recentrifugation. In two experiments, LDL were subfractionated by equilibrium density-gradient centrifugation (9). Isolation of LDL was started within 1 day after blood was drawn from the donors, who had fasted overnight. Lipoprotein-deficient human serum (LPDS) was prepared by ultracentrifugation (11). The LDL were radiolabeled by the iodine monochloride procedure (11). Unlabeled and radiolabeled lipoproteins were dialyzed against 0.15 M NaCl/15 mM EDTA, pH 7.4, before use in cell culture experiments.

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Abbreviations: LDL, low density lipoprotein(s); ^{125}I-LDL, ^{125}I-labeled LDL; apo (prefix), apolipoprotein; FH, familial hypercholesterolemia; DMEM, Dulbecco's modified Eagle's medium; LPDS, lipoprotein-deficient human serum.

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ApoB-100 and its thrombolytic fragments (13) were analyzed by one-dimensional NaDodSO$_4$/polyacrylamide slab gel electrophoresis using reduced samples (14). The LDL were partially delipidated in diethyl ether (10). Protein and cholesterol concentrations were determined by the method of Lowry *et al.* (15) and with a spectrophotometric assay kit (Boehringer Mannheim), respectively. Phospholipids were measured by the procedure of Bartlett (16).

Cell Culture. Most of these studies used normal human fibroblasts derived from a preputial specimen from a normal human infant and cultured as monolayers in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (12, 13). The human fibroblast cell strain GM483, derived from a FH heterozygote, was obtained from the NIGMS Human Genetic Mutant Cell Repository (Camden, NJ). Fibroblasts were also obtained from a skin biopsy of patient G.R. and an age-matched normal control (17). Experiments were performed on confluent monolayers of fibroblasts that had been plated (35,000 cells per 35-mm tissue culture dish) 7 days previously. Cells were incubated with DMEM containing 10% LPDS for 2 days before the tissue culture experiment. For lipoprotein competition experiments, normal human ^{125}I-labeled (^{125}I-) LDL protein (2 μg/ml for 4°C experiments, 5 μg/ml for 37°C experiments) was added along with increasing concentrations of unlabeled LDL to DMEM containing 10% LPDS. The cells were incubated for 2 hr at 4°C or 5 hr at 37°C. The surface-bound radioactivity was determined for the 4°C binding experiments, or the amount of cellular uptake and proteolytic degradation was determined for the 37°C binding experiments (11, 12). The amount of unlabeled LDL needed to displace 50% of the ^{125}I-labeled ligand was calculated by linear regression analysis of the logarithm of concentration (μg of protein/ml) vs. probits. Probits were read from a probit transformation table (18).

Direct cell-surface binding of ^{125}I-LDL was measured after a 4-hr incubation at 4°C (11, 12). Receptor binding data were linearized by Scatchard analysis (12, 19), and the equilibrium dissociation constant (K_d) and the maximum amount of LDL bound at receptor saturation (B_{max}) were determined (11). To determine the amount of cholesteryl [^{14}C]oleate formation, normal human fibroblasts were incubated for 18 hr at 37°C with 0.2 mM [^{14}C]oleate/albumin in DMEM (11, 12) in the absence or presence of various concentrations of LDL. Cholesteryl [^{14}C]oleate was isolated by TLC and measured by liquid scintillation counting, using [^{3}H]cholesteryl oleate as an internal standard (11, 12).

RESULTS

In vivo turnover studies of LDL from several hypercholesterolemic subjects had suggested that in some the LDL were poor ligands for apoB,E(LDL) receptors (8). To test this postulate directly, LDL were isolated from five subjects with apparently abnormal LDL and tested for their ability to compete with normal human ^{125}I-LDL for binding to apoB,E-(LDL) receptors on normal human fibroblasts. In only one of these subjects was the receptor binding of LDL defective (G.R.; see figure 2*D* and table II of ref. 8, subject 15). As shown in Fig. 1, the unlabeled LDL from subject G.R. were not as effective as normal LDL in competing with normal ^{125}I-LDL for receptor binding. In seven separate competition experiments performed at 4°C, three separate preparations of G.R. LDL were compared with 15 preparations of LDL from nine normal individuals. A 50% displacement of normal ^{125}I-LDL from human fibroblasts required 9.3 ± 3.5 μg of G.R. LDL protein per ml (±SD) compared with only 3.0 ± 1.6 μg of normal LDL protein per ml.

Further, the G.R. LDL were clearly less effective than normal unlabeled LDL in competing with normal ^{125}I-LDL for receptor-mediated uptake and degradation in human fibroblasts at 37°C (Fig. 2). Compared with normal LDL, about 2.6 times more G.R. LDL was required to inhibit 50% of the receptor-mediated cellular uptake and degradation of normal ^{125}I-LDL.

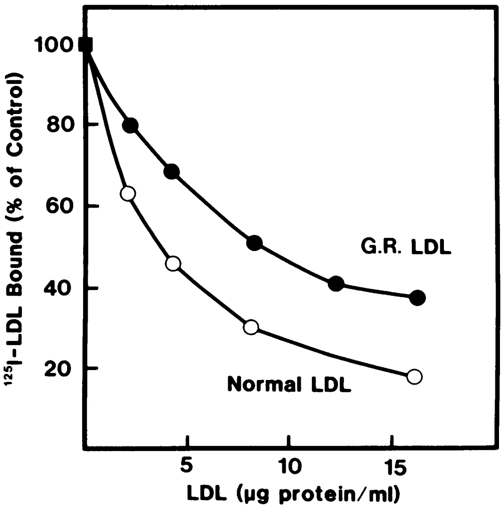

FIG. 1. Ability of unlabeled LDL from a normal control subject and from subject G.R. to compete with normal ^{125}I-LDL for binding to apoB,E(LDL) receptors on normal human fibroblasts. One milliliter of DMEM containing 10% LPDS, 2 μg of normal ^{125}I-LDL protein, and the indicated concentrations of unlabeled G.R. LDL or normal LDL were added to 35-mm culture dishes containing normal human fibroblasts. After a 2-hr incubation at 4°C, the amount of ^{125}I-LDL bound was measured. The 100% control value was 150 ng/mg of cellular protein.

Determination of the amount of [^{14}C]oleate incorporated into cellular cholesteryl esters serves as a measure of lipoprotein delivery of cholesterol to cells (11). Normal human fibroblasts were incubated at 37°C for 18 hr in the presence of [^{14}C]oleate with various concentrations of LDL from either two normal controls or subject G.R., and cellular cholesteryl ester formation was measured (Fig. 3). The G.R. LDL were less effective than normal LDL in promoting cholesteryl [^{14}C]oleate formation.

Similar studies were carried out in four first-degree relatives of G.R. Two brothers (W.R. and C.R.) were found to have the same defect as G.R. One brother (Sta.R.) and G.R.'s son (Ste.R.) appeared to be unaffected. Increased total cholesterol concentrations were found in plasma from the affected family members (Table 1). Two tentative conclusions can be drawn from these data. First, the binding defect appears to have a codominant mode of transmission; second, the affected family members appear to be heterozygous for this defect, which most likely resides in an apoB-100 allele. The basis for these conclusions is that there is only one apoB-100 molecule per LDL particle (20). Therefore, in a heterozygous state it is not possible for normal apoB-100 to compensate totally for the defective form and to produce a normal phenotype. If the proband (G.R.) were a homozygote, then his son (Ste.R.) would be at least an obligate heterozygote whose LDL would have partial receptor binding activity, which could be detected by the quantitative receptor binding assay. Therefore, because the son had completely normal binding activity, the father must be heterozygous for this defect. Consequently, the LDL from the affected family members should consist of two populations of particles: one with normal receptor binding, and the other with defective receptor binding.

Equilibrium density-gradient centrifugation was used to subfractionate the LDL from two affected family members and from a normal control (Table 2). The LDL from the

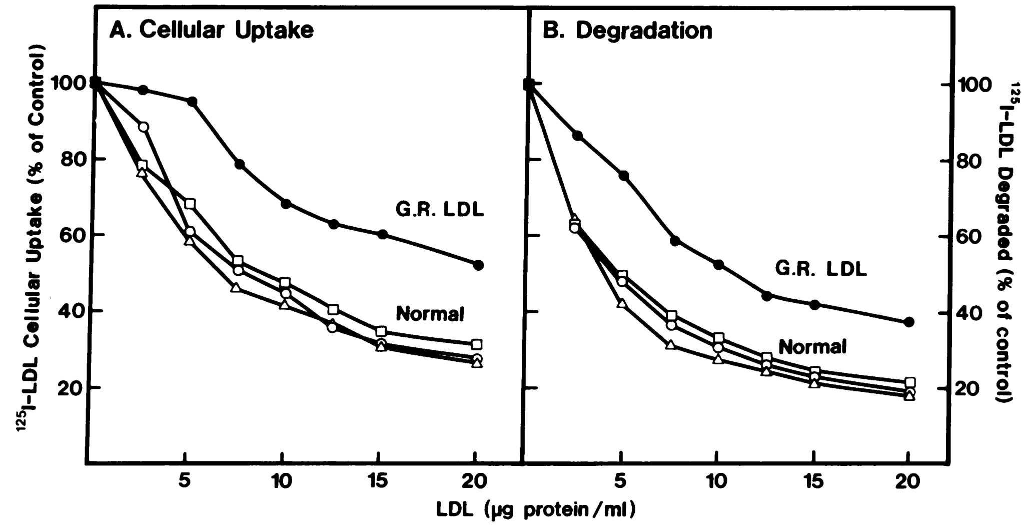

FIG. 2. Ability of LDL from subject G.R. and from three normal individuals to compete with normal ^{125}I-LDL for receptor-mediated cellular uptake (*A*) and proteolytic degradation (*B*). One milliliter of DMEM containing 10% LPDS, 5 μg of normal ^{125}I-LDL protein, and the indicated concentrations of unlabeled G.R. LDL or normal LDL were added to 35-mm dishes containing normal human fibroblasts. After a 5-hr incubation at 37°C, the amount of ^{125}I-LDL bound, internalized, and degraded was measured. The 100% control values for cellular uptake and degradation were 520 and 2200 ng/mg of cellular protein, respectively.

normal subject (compared with W.R.) produced 50% inhibition of ^{125}I-LDL binding at 2.9 μg of LDL protein per ml, and concentrations of the subfractions needed for 50% inhibition ranged from 2.7 to 4.6 μg/ml. On the other hand, the concentrations of unfractionated LDL from W.R. and from G.R. necessary for a 50% inhibition were 9.1 and 5.8 μg/ml, respectively (Table 2). In contrast to the results obtained with the subfractions of LDL from the normal subject, the subfractions from the affected subjects produced 50% inhibition of ^{125}I-LDL binding over a wide range of concentrations [7.0–18.3 μg/ml (W.R.) and 3.9–15.1 μg/ml (G.R.)]. In both subjects the concentration of subfraction needed for 50% inhibition of binding increased with increasing density of the subfractions. These results indicate that unfractionated LDL include subfractions enriched in both functionally normal and functionally abnormal populations of LDL—i.e., subfractions with either higher or lower receptor binding activity than the total unfractionated LDL.

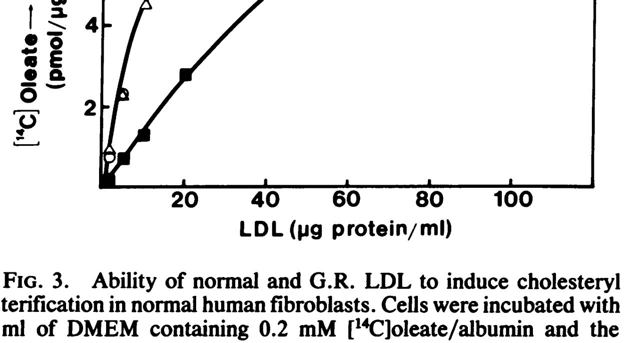

FIG. 3. Ability of normal and G.R. LDL to induce cholesteryl esterification in normal human fibroblasts. Cells were incubated with 1 ml of DMEM containing 0.2 mM [^{14}C]oleate/albumin and the indicated concentrations of G.R. LDL or normal LDL. After an 18-hr incubation at 37°C, the amount of cholesteryl [^{14}C]oleate was measured.

Because binding of LDL to the apoB,E(LDL) receptor is mediated by apoB-100, it is very likely that the structural abnormality of the affected LDL resides in apoB-100. To rule out the possibility that defective receptor binding was directly related to lipid composition, the lipid content of normal and mutant LDL was markedly altered by partial delipidation with ether (10). Even though partial delipidation removed >95% of the total cholesterol and triacylglycerols from both normal and mutant LDL, both still exhibited receptor binding activity essentially identical to that of their untreated counterparts (Fig. 4). Thus, removing most of the lipid from mutant LDL did not alter their binding activity, which was still only about one-third that of normal LDL.

In both affected and normal family members, the chemical composition of LDL was essentially the same (Table 3). Likewise, there were no detectable differences in the size and shape of their LDL as determined from electron micrographs of negatively stained LDL and nondenaturing gradient-gel electrophoresis (data not shown). Further, based on migration in $NaDodSO_4$/polyacrylamide gels, the molecular weight of apoB-100 from all family members appeared to be identical to that of normal apoB-100 (M_r 550,000). Similarly, the apparent molecular weights of the thrombolytic fragments of apoB-100 from the affected subjects appeared to be identical to those previously observed for normal apoB-100 (data not shown) (13).

Table 1. Characterization of G.R. family members

Family member	Age, years	Plasma cholesterol, mg/ml	Coronary heart disease	LDL IC_{50},* μg/ml
Affected				
G.R. (proband)	68	311	−	9.3 ± 3.5
W.R. (brother)	72	305	+	6.9
C.R. (brother)	70	247	+	7.1
Unaffected				
Sta.R. (brother)	64	194	+	2.2
Ste.R. (son)	34	181	?	3.0

*Concentration of LDL required for 50% inhibition of ^{125}I-LDL binding to normal human fibroblasts; for LDL from normal individuals, the value was 2.2 μg of LDL protein per ml.

Table 2. Ability of subfractions of binding-defective LDL to compete with normal ^{125}I-LDL for binding to fibroblast receptors

	Volume, ml	Density, g/ml	LDL IC$_{50}$,* μg/ml	
			Patient LDL	Normal LDL
		Patient W.R.		
Total LDL	—	—	9.1	2.9
Subfraction				
1	1.0	1.024	7.0	2.7
2	1.0	1.026	7.8	3.5
3	0.5	1.030	10.3	3.7
4	0.5	1.032	10.9	3.3
5	0.5	1.035	13.1	3.4
6	0.5	1.038	18.0	4.3
7	1.0	1.044	18.3	4.6
		Patient G.R.		
Total LDL	—	—	5.8	1.7
Subfraction				
1	1.0	1.024	3.9	ND
2	1.0	1.028	7.9	ND
3	0.5	1.032	11.2	ND
4	0.5	1.035	11.2	ND
5	0.5	1.038	15.1	ND

*Defined in footnote to Table 1. ND, not determined.

Table 3. Composition of LDL from G.R. family members

	Percent composition (wt/wt)			
Subject	Protein	Cholesterol	Triacylglycerol	Phospholipid
G.R.*	24.8	43.0	3.6	28.6
W.R.*	25.1	43.0	4.3	27.6
C.R.*	22.2	42.6	3.7	31.5
Sta.R.	25.0	43.8	5.3	25.9
Ste.R.	23.6	44.1	4.3	28.0
Normal (control)	22.5	43.6	4.2	29.7

*Affected subjects whose LDL bind abnormally to apoB,E(LDL) receptors.

To verify that the proband was not a classic FH heterozygote, skin fibroblasts were cultured from G.R. and an age-matched control. The number of apoB,E(LDL) receptors and the affinity of the receptors for normal LDL were measured by equilibrium binding experiments using normal ^{125}I-LDL. The numbers of apoB,E(LDL) receptors per cell (number of LDL particles bound at receptor saturation) for the cultured fibroblasts from G.R., the normal control, and a FH heterozygote (GM483) were 134,000, 111,000, and 44,000, respectively. In addition, the apoB,E(LDL) receptors on the G.R. fibroblasts had normal affinity for normal LDL ($K_d = 3.4 \times 10^{-9}$ M, 2 μg of protein per ml).

DISCUSSION

This study describes functionally abnormal LDL that bind poorly to apoB,E(LDL) receptors. As determined in different types of tissue culture assays, LDL from subject G.R. exhibit defective binding to apoB,E(LDL) receptors on normal human fibroblasts. Examination of the binding ability of LDL from four first-degree relatives demonstrated that two brothers had functionally abnormal LDL and a brother and a son had normal LDL. Because LDL binding to the apoB,E(LDL) receptor is mediated by apoB-100 (2), the defective receptor binding is probably due to a structural variation in apoB-100. This conclusion is supported by the observation that the defective receptor binding remained after the mutant LDL were partially delipidated. Thus, the defective binding of the proband's LDL to the apoB,E(LDL) receptor is probably the result of a mutation in one of the copies of the apoB-100 gene. We have designated this abnormality familial defective apoB-100.

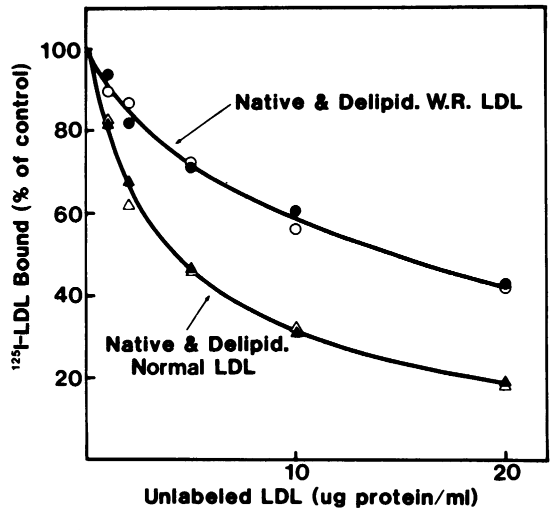

FIG. 4. Comparison of the effect of partial delipidation on the ability of W.R. and normal LDL to compete with normal ^{125}I-LDL for receptors on normal human fibroblasts. Cells were incubated with 1 ml of DMEM containing 10% LPDS, 2 μg of normal ^{125}I-LDL protein, and the indicated concentrations of unlabeled native (filled symbols) and partially delipidated (open symbols) LDL. After a 2-hr incubation at 4°C, the amount of ^{125}I-LDL bound to the cells was measured. The 100% control value was 110 ng/mg of cellular protein.

The familial studies suggest that the proband and the affected family members are heterozygotes, possessing a normal and a mutant apoB-100 allele. Since there is only one apoB-100 molecule per LDL particle (20), the LDL of G.R. and other affected kindred undoubtedly represent a mixture of LDL that bind normally and LDL that bind abnormally to the apoB,E(LDL) receptors. The finding that LDL in fractions of higher density (1.038–1.044 g/ml) had markedly impaired binding in comparison with LDL in more buoyant fractions is consistent with the possibility that the affected individuals are heterozygotes who have subpopulations of both normal and receptor-defective LDL. If so, then the abnormal LDL should exist in the plasma in higher concentration than the normal LDL because the abnormal LDL are not as effectively cleared by a receptor-mediated mechanism (21, 22). In addition, the longer retention of the abnormal LDL in plasma may increase the extent of their metabolic conversion to denser LDL subspecies, enriching these fractions with abnormal LDL.

The inefficient clearance of the mutant LDL appears to result in the accumulation of LDL in the plasma and to cause the hypercholesterolemia observed in the proband. As a first approximation, the situation is similar to that of patients with heterozygous FH. In both situations the accumulation of LDL in the plasma is a consequence of defective ligand–receptor interactions. The defect involves half of the receptors in FH heterozygotes, and half of the ligands in heterozygotes with familial defective apoB-100. However, there are important differences that may explain why the plasma cholesterol levels of affected G.R. family members (247–311 mg/dl) were somewhat lower than the cholesterol levels typically found in FH heterozygotes (350–375 mg/dl). FH disrupts receptor-mediated uptake of both apoB-100- and apoE-containing lipoproteins, including apoE-mediated uptake of very low density lipoproteins and intermediate density lipoproteins and apoB-mediated uptake of LDL (23, 24). In contrast, in the case of familial defective apoB-100, the primary effect would be a decreased clearance of apoB-containing LDL and may not involve intermediate density lipoprotein and very low density lipoprotein particles that contain apoE as well as apoB-100. It is reasonable to postulate that this accounts for the somewhat lower plasma cholesterol levels in the affected G.R. family members compared with FH heterozygotes.

The frequency of occurrence of primary hypercholesterolemia resulting from familial defective apoB-100 is not known; however, only a small percentage of patients with primary hypercholesterolemia possess functionally defective apoB,E(LDL) receptors, and many genetic and environmental factors (6, 25) undoubtedly play a role. Vega and Grundy (8) described five patients with retarded LDL clearance, but only one (G.R.) was found to possess LDL defective in binding to apoB,E(LDL) receptors by the fibroblast *in vitro* binding assay.

Recently, Young *et al.* (26, 27) reported a genetic abnormality in LDL in which a grossly abnormal (truncated) apoB-100 was found. In contrast to their finding, no major physical or chemical abnormalities could be detected in the lipids or in the apoB-100 of the LDL of the current proband or the affected brothers. Polyacrylamide gel electrophoresis of the whole apoB-100 or its thrombolytic fragments revealed no gross size abnormalities. The molecular nature of the structural defect ultimately will be revealed by analysis of the gene that produces the defective-binding apoB-100. The structure of the normal human apoB-100 gene was elucidated recently (28).

Knowledge of the structural abnormality that causes the defective receptor binding of the apoB-100 in this family is likely to help define the receptor-binding domain of apoB-100. In previous studies (for review, see ref. 2) of the receptor-binding domain of apoE, the elucidation of the structure of apoE mutants that interact poorly with the apoB,E(LDL) receptor proved valuable. All of the apoE mutants that were defective in receptor binding had neutral amino acids substituted for basic amino acids, and the substitutions were clustered in the center of the apoE molecule. Other approaches confirmed that the central region of apoE is responsible for direct interaction with the receptor—i.e., it contains the receptor-binding site (2). Although the receptor-binding domain of apoB-100 has not been precisely located, several lines of evidence have focused attention on a region of apoB-100 near residue 3249 (29). Two sequences in this region are enriched in basic amino acids, reveal a homology to the receptor-binding domain of apoE, and are in the vicinity of the epitopes of monoclonal antibodies that bind heparin with high affinity (30) and inhibit LDL binding to the receptors (31). As with apoE, identification of the amino acid substitutions, deletions, or additions within apoB-100 that alter receptor-binding activity should help to define the receptor-binding domain of apoB-100.

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